Appl Microbiol Biotechnol (2003) 62:110–123
DOI 10.1007/s00253-003-1341-4

MINI-REVIEW

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Evolution of catabolic pathways for synthetic compounds: bacterial pathways for degradation of 2,4-dinitrotoluene and nitrobenzene

Received: 6 February 2003 / Revised: 8 April 2003 / Accepted: 11 April 2003 / Published online: 15 May 2003

Abstract The pathways for 2,4-dinitrotoluene (2,4-DNT) and nitrobenzene offer fine illustrations of how the ability to assimilate new carbon sources evolves in bacteria. Studies of the degradation pathways provide insight about two principal strategies for overcoming the metabolic block imposed by nitro- substituents on aromatic compounds. The 2,4-DNT pathway uses novel oxygenases for oxidative denitration and subsequent ring-fission. The nitrobenzene pathway links facile reduction of the nitro-substituent, a novel mutase enzyme, and a conserved operon encoding aminophenol degradation for mineralization of nitrobenzene. Molecular genetic analysis with comparative biochemistry reveals how the pathways were assembled in response to the recent appearance of the two synthetic chemicals in the biosphere.

Introduction

Bacterial metabolism is the cornerstone supporting the global carbon cycle. Bacteria have a remarkably diverse collection of degradative abilities that enable the recycling of nearly all known organic material. Extensive research on the biochemistry, molecular genetics, and microbial ecology of bacteria during the past 50 years has allowed a nearly complete understanding of the relevant metabolic processes. Much of the work has focused on catabolic pathways for natural organic compounds, including hydrocarbons. The biochemistry and molecular genetics of the pathways for the naturally occurring compounds are relatively well understood and indicate that the pathways are highly evolved. For example, the kinetics of the reactions indicate that the enzymes are well suited for the individual steps, the genes encoding sequential steps are closely linked in operons without superfluous genetic material to interfere with expression of structural genes, and the expression of the catabolic genes is regulated to avoid metabolic bottlenecks and respond to changing conditions. The efficiency is testament to the millions and even billions of years that bacteria have been exposed to the natural organic compounds and the evolution that has been driven by natural selection.

In the past century, modern chemistry has produced a multitude of synthetic organic compounds that are not found in nature. Nonetheless, bacteria able to degrade the xenobiotic compounds are often found in the environment and, in many instances, it is clear that the bacteria recently evolved the capability in response to potential new growth substrates. Examination of the molecular biology and biochemistry of the pathways for synthetic compounds reveals evidence of the recent and ongoing evolution. Such evidence includes: remnants of genetic material from lateral gene transfer, scattered organization of the genes encoding the enzymes, primitive or inefficient regulation of enzyme synthesis, and poorly adapted degradative enzymes (high K_m, low K_{cat}, low catalytic specificity). Study of the relatively primitive pathways reveals the strategies that bacteria use to evolve new capabilities and provides insight about the origins of the catabolic steps.

Several studies present molecular genetic evidence to define how catabolic pathways for synthetic compounds evolved. The chlorobenzene pathways from *Pseudomonas* sp. strain P51 (Werlen et al. 1996) *Burkholderia* sp. strain PS12 (Beil et al. 1999), and *Ralstonia* sp. strain JS705 (van der Meer et al. 1998; Müller et al. 2003) are explicit examples. In all cases, the pathway appears to have come about via transposon-mediated insertion of a hydroxylating dioxygenase with a broad substrate range and dihydrodiol dehydrogenase gene cluster next to an operon encoding a modified ortho- ring-cleavage pathway for chlorocatechol degradation. In *Pseudomonas* sp. strain P51, the transposon containing the genes for the hydroxylating dioxygenase and diol dehydrogenase also encodes remnants of the hydrolase and ring-fission dioxygenase

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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 FEB 2003		

4. TITLE AND SUBTITLE
Evolution of Catabolic Pathways for Synthetic Compounds Bacterial Pathways for Degradation of 2,4-dinitrotoluene and Nitorbenzene

5a. CONTRACT NUMBER
F08637-98-C-6002

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
61102F

6. AUTHOR(S)
Glenn Johnson; Jim Spain

5d. PROJECT NUMBER
2312

5e. TASK NUMBER
C4

5f. WORK UNIT NUMBER
2312C401

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Air Force Research Laboratory,Materials and Manufacturing Directorate,139 Barnes Drive, Suite 2,Tyndall Air Force Base,FL,32403-5323

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
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15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**		**15**	

for methylcatechol degradation (Werlen et al. 1996). The authors suggested that inactivation of the methylcatechol pathway resulting from transposition avoids misrouting of chlorocatechol during degradation of chlorobenzene (Werlen et al. 1996). In the case of strain JS705, the authors provide insight not only about how the pathway evolved but also about when and where within a contaminated ecosystem (van der Meer et al. 1998; Müller et al. 2003). The in situ evolution of chlorobenzene catabolism in strain JS705 provides a parallel in nature to previous laboratory construction of hybrid strains for chloroaromatic compound degradation (Reineke 1998) and an interesting analogy to the evolution of haloalkane catabolism (Poelarends et al. 2000).

The pathway for pentachlorophenol metabolism also evolved via a recruitment strategy. Combination of a glutathione-dependent dehalogenase with a 4-chlorophenol catabolic pathway resulted in the expanded catabolism (Anandarajah et al. 2000). In the first step of the pathway, the non-specific flavin monooxygenase from the chlorophenol pathway catalyzes hydroxylation of pentachlorophenol to tetrachlorobenzoquinone, which is reduced to yield tetrachlorohydroquinone (Dai et al. 2003). The recently recruited dehalogenase then catalyzes two consecutive dehalogenations to produce 3,5-dichlorohydroquinone, which serves as the ring-fission substrate (Xu et al. 1999).

A third intriguing example comes from the pathway for the widely used herbicide, atrazine. The structure of atrazine consists of an *s*-triazine ring with chlorine and N-linked ethylamino and isopropylamino substituents on the carbons of the ring. A series of three novel hydrolase reactions removes the ring substituents and replaces them with hydroxyl groups to yield cyanuric acid, a compound commonly degraded by soil microbes (Eaton and Karns 1991; de Souza et al. 1998). The genes for the hydrolase enzymes in the atrazine pathway all arose from the amidohydrolase superfamily, but their sequences are so different that it appears each of the hydrolases in the pathway evolved independently from different ancestors. What is truly remarkable about the evolution of atrazine metabolism is that the three disparate hydrolase steps came together and the system was dispersed worldwide as a result of the selective pressure exerted by atrazine application during the past four decades (Wackett and Hershberger 2001).

Nitro-substituted organic compounds pose a distinct challenge to bacterial metabolism. Exposure has been limited, because there are only a few naturally occurring nitro-substituted compounds. Nitroarenes can result from photochemical transformation of polyaromatic hydrocarbons in the atmosphere (Crawford 1993). Pyrrolnitrin and chloramphenicol, two antibiotics that are synthesized by *Pseudomonas* spp and *Streptomyces* spp, respectively, are nitroaromatic compounds (Ahmed and Vining 1983; Kirner et al. 1998). Lastly, plants such as crown vetch and milk vetch (*Astragallus* spp) synthesize nitroglycosides as part of their defense repertoire (Wackett and Hershberger 2001). Although little is known about the degradation of the biogenic nitroarenes, the examples are evidence that the nitro functional group is not xenobiotic; but bacteria have not been exposed to a wide variety of nitro-substituted molecules, as is the case with other recalcitrant compounds such as naturally occurring halogenated organic compounds (Harper 1994; Gribble 1998).

Virtually all living organisms have the potential to catalyze the reduction of nitro groups on aromatic compounds. The net positive charge on the nitrogen atom makes it very susceptible to reduction by a number of redox enzymes. The cometabolic processes can be detrimental to the cell (Spain 1995; Esteve-Nuñez et al. 2001; Riefler and Smets 2002), the sequential reduction requires a substantial input of electrons, and it can yield highly reactive intermediates (Ahmad and Hughes 2002). Accordingly, it is appropriate to address any questions of bacterial evolution through study of organisms that completely degrade the nitroarenes and thus obtain a selective advantage from the process.

Although environmental exposure to nitroarenes has been limited until recently, bacteria developed strategies for their assimilation remarkably quickly. In some instances, the compounds are transformed and used as a source of nitrogen (Vorbeck et al. 1994) or as a terminal electron acceptor (Esteve-Nuñez and Ramos 1998; Esteve-Nuñez et al. 2000), but entire pathways that allow nitroarenes to serve as carbon, energy, and nitrogen sources also exist and are the focus of this review.

Bacteria use a variety of strategies to deal with the nitro substituent on the ring. Oxygenase attack is often the first step in the aerobic degradation of aromatic compounds (Dagley 1986). Both monooxygenase and dioxygenase enzymes are used to catalyze the replacement of the nitro group with hydroxyl groups as the first step in the degradation of a variety of nitroaroamatic compounds (Nishino et al. 2000b). Such reactions serve not only to eliminate the nitro group, but also activate the ring for subsequent breakdown. The facile reduction of the nitro group is exploited in some pathways where unusual catabolic enzymes intercept the reduced intermediates and channel them into productive central pathways (Nishino et al. 2000b). The phenylhydroxylamine that results from partial reduction of the nitro group can be transformed to the corresponding catechol and ammonia by hydroxylaminolyase or undergo mutase-catalyzed rearrangement to yield an aminophenol (AP). Another strategy, used for polynitroaromatic compounds such as picric acid, begins with reduction of the aromatic ring to yield a hydride–Meisenheimer complex. Subsequent protonation of the nitro-bearing carbon leads to nitrite elimination and re-aromatization of the ring (Ebert et al. 1999, 2001).

The catabolic pathways and basic biochemistry for the degradation of a number of nitroaromatic compounds are well understood. Recent reviews provide detailed overviews of nitroarene degradation with respect to bacterial metabolism (Blotevogel and Gorontzy 2000; Nishino et al. 2000b; Esteve-Nuñez et al. 2001; Heiss and Knackmuss 2002) and the potential for biologically based

remediation of nitroarene- and explosives-contaminated environments (Peres and Agathos 2000; Rodgers and Bunce 2001; Dua et al. 2002; Rieger et al. 2002; Snellinx et al. 2002). The understanding of the genetic basis for nitroarene metabolism is still emerging. The genes encoding the pathways or individual steps in pathways for the degradation of a number of compounds including: 4-nitrophenol (Bang and Zylstra 1996, 1997; Perry and Zylstra 1999), 4-nitrobenzoate (accession numbers AF187879, AF187880 Yabannavar and Zylstra 1995; Hughes and Williams 2001), 2,4-dinitrophenol (Walters et al. 2001), 2,4,6-trinitrophenol (Heiss et al. 2002), nitrobenzene (He et al. 1998; Davis et al. 1999, 2000; Park and Kim 2000, 2001; Lessner et al. 2002), 2-nitrotoluene (Parales et al. 1996), 4-nitrotoluene (James and Williams 1998; James et al. 2000), and 2,4-dinitrotoluene (2,4-DNT; Suen and Spain 1993; Haigler et al. 1996; Haigler et al. 1999; Johnson et al. 2000, 2002) have been cloned and characterized. Particularly interesting aspects of the evolution of dinitrophenol and trinitrophenol degradation are emerging from the studies. The polynitrophenol pathways display evidence for regulatory mechanisms that suggest developing pathways (Walters et al. 2001; Heiss et al. 2002). Other molecular engineering efforts have developed bacterial strains that utilize nitroarenes through hybrid pathways (Duque et al. 1993; Michán et al. 1997). It is not the aim of this review to consider all the molecular genetic studies of nitroarene degradation. Instead, the review discusses two pathways in which all steps from initial transformation of the nitroarene to central metabolic compounds are characterized and complete sequence information is available: the oxidative pathway for 2,4-DNT and the partially reductive pathway for nitrobenzene. The discussion focuses on presenting the interpretation of molecular genetic characterization and comparative biochemistry that define how the pathways for 2,4-DNT and nitrobenzene were assembled from potential progenitors for individual enzymes in the degradative pathway.

In the examples above and other work discussed here, the evidence used to determine the evolutionary origins of a metabolic pathway comes from analysis of nucleotide sequences, analogies between reaction mechanisms in different pathways, and characterization of the structure of enzymes in the pathways. The organization of the genes encoding a pathway reveals some insight about its origin and perhaps its maturity, i.e., a compact regulated operon suggests a highly evolved degradative pathway, while a single gene product relevant to the new catabolic pathway surrounded by inactivated genes or genes encoding unrelated steps is indicative of recently evolved, non-optimized catabolism. Further analysis of the sequence outside the coding region can indicate lateral gene transfer by the presence of transposon-associated elements or their remnants. Such analysis can provide considerable confidence regarding the origin of the genes that encode the pathway and, in many cases, how they were assembled. Sequence analysis alone, however, sheds little light on when, where, or why a set of reactions was assembled to provide the new catabolic route. A considerably broader understanding, including biochemistry, physiology, and microbial ecology, is needed before evolution of bacterial metabolism can be understood fully or predicted with confidence.

2,4-DNT degradation: three novel oxygenases

Bacterial degradation of 2,4-DNT proceeds by an oxidative pathway that yields nitrite, pyruvate, and propionyl-CoA (Fig. 1; Johnson et al. 2002). Bacterial strains able to grow using 2,4-DNT have been isolated from numerous contaminated ecosystems around the world and all appear to use the same pathway for 2,4-DNT degradation (Nishino et al. 2000a, 2000b). The two oxygenases that catalyze the first steps in the pathway remove the two nitro substituents and yield 2-hydroxy-5-methyl-benzoquinone. The hydroxyquinone is then reduced to the ring-fission substrate, 2,4,5-trihydroxytoluene, which undergoes dioxygenase-catalyzed *meta*-cleavage and subsequent degradation to compounds that enter dissimilatory pathways of the cell. Characterization of the molecular biology of 2,4-DNT degradation revealed that the pathway was assembled from three modules centered around the oxygenase genes (Fig. 1).

The catalytic specificity and substrate preferences of the three oxygenases in the 2,4-DNT pathway are unusual, compared to oxygenases from pathways for aromatic hydrocarbon degradation. The first steps in the pathway hydroxylate the ring and lead to elimination of the nitro substituents as anions. The oxidative removal of the nitro substituents from the ring is analogous to the removal of other anionic leaving groups, such as halogen, carboxyl, and sulfonate substituents (Knackmuss 1996). The ring-fission step in the 2,4-DNT pathway is analogous to the *meta*- ring-fission of trihydroxy-aromatic compounds found in the resorcinol degradation pathway (Chapman and Ribbons 1976a). The ring-fission substrates are different trihydroxytoluene isomers, but the hydroxyl substituents have the same relative orientation on the aromatic ring.

The 2,4-DNT dioxygenase is among a group of three-component enzyme systems comprising a flavoprotein NAD(P)H reductase, a Rieske-iron containing ferredoxin, and a terminal oxygenase component consisting of two non-identical subunits (Harayama et al. 1992). The oxygenase component is the catalytic part of the enzyme. The larger subunit (α) contains a Rieske iron center and a mononuclear iron-binding site, which activates the oxygen molecule for the reaction (Suen and Gibson 1993; Jiang et al. 1996). The α-subunit structure determines the catalytic specificity of enzymes in the group (Erickson and Mondello 1992; Gibson et al. 1993; Jiang et al. 1996; Kimura et al. 1997; Mondello et al. 1997; Parales et al. 1998a, 1998b, 2000; Sakamoto et al. 2001; Yu et al. 2001). This influence was confirmed by testing the catalytic specificity and substrate range of altered dioxy-

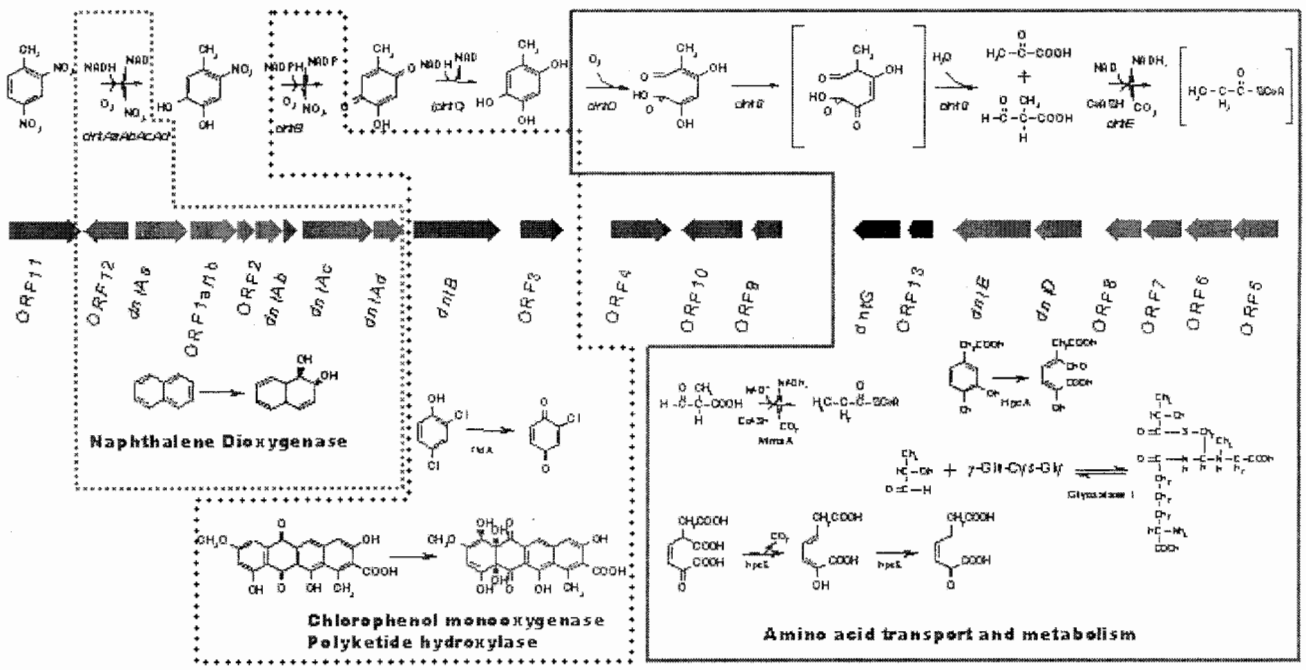

Fig. 1 Organization and evolution of the 2,4-dinitrotoluene (2,4-DNT) gene cluster in *Burkholderia cepacia* R34. The upper panel outlines the catabolic pathway for 2,4-DNT. Gene designations for the enzymes are shown below the reaction arrows. The central portion of the figure displays a map of the region on pJS311 that encodes the 2,4-DNT pathway in strain R34 (Johnson et al. 2002). The solid arrows correspond to open reading frames (ORFs) that were identified from sequence analysis. ORFs with dnt-designations indicate gene products with assigned functions in the 2,4-DNT pathway. Coding regions with ORF designations indicate gene products and hypothetical proteins identified from sequence analysis, but whose physiological function has not been confirmed. Reactions shown below the physical map depict reactions catalyzed by putative homologues of enzymes in the 2,4-DNT pathway

genases made using hybrid polypeptides, site-directed mutagenesis, and shuffling mutagenesis.

The multicomponent dioxygenases for aromatic compounds separate into three phylogenetic groups: aromatic acid dioxygenases (benzoate, toluate, 2,4-dichlorophenoxy acetic acid), benzenoid dioxygenases (benzene, toluene, biphenyl, chlorobenzene), and naphthalene dioxygenases (naphthalene, polyaromatic hydrocarbons, benzothiophene) (Nam et al. 2001). The nitroarene dioxygenases are closely related to naphthalene dioxygenases (Parales et al. 1996; Suen et al. 1996; Johnson et al. 2002; Lessner et al. 2002) (Fig. 2a). The greatest sequence identity is shared with the subgroup of naphthalene dioxygenases, coupled with gentisate pathways exemplified by *Ralstonia* sp. strain U2 (Fuenmayor et al. 1998) and *Comamonas testosteroni* GZ42 (Goyal and Zylstra 1997). The α-subunit from a recently isolated polycyclic aromatic hydrocarbon-degrading strain, *C. testosteroni* H, also appears to be within the subgroup. Further detail is needed to confirm its relatedness, since only a portion of the dioxygenase operon sequence is available (accession AAF727976) and the expression of the naphthalene pathway for the strain was not reported (Moser and Stahl 2001).

The nitroarene dioxygenases share little sequence identity (<35%) with toluene dioxygenases and tetrachlorobenzene dioxygenase, even though there is some catalytic overlap between the enzymes. The tetrachlorobenzene dioxygenases from *Burkholderia* sp. strains PS12 and PS14 catalyze the conversion of 2,4,5-trichloronitrobenzene to nitrite and 3,4,6-trichlorocatechol, but the specific activity was three orders of magnitude less than for the physiological substrate, 1,2,4,5-tetrachlorobenzene (Sander et al. 1991). The nitrobenzene dioxygenase catalyzes dioxygenation of toluene (Lessner et al. 2002), whereas naphthalene dioxygenase attacks the methyl group of toluene (Lee and Gibson 1996). It remains a mystery why the nitroarene and prototypical naphthalene dioxygenases, which have nearly identical oxygenase subunit structures, catalyze different reactions with monocyclic substrates, such as toluene, but similar dioxygenation of naphthalene.

The similarity in the organization of the operons encoding the 2,4-DNT dioxygenases and the naphthalene dioxygenase found in strain U2, like the sequence identity shared by the α-subunits, indicates that the enzymes share a recent common ancestor (Fuenmayor et al. 1998; Parales 2000; Johnson et al. 2002). The dioxygenase operon from strain U2 differs from the prototypical naphthalene dioxygenase from strain 9816 in that it includes two additional genes that encode a terminal oxygenase component for salicylate hydroxylase (Fuenmayor et al. 1998; Zhou et al. 2002). Remnants of the salicylate hydroxylase genes are found in all the operons that encode nitroarene dioxygenases studied to date. The presence of inactivated or partially deleted salicylate

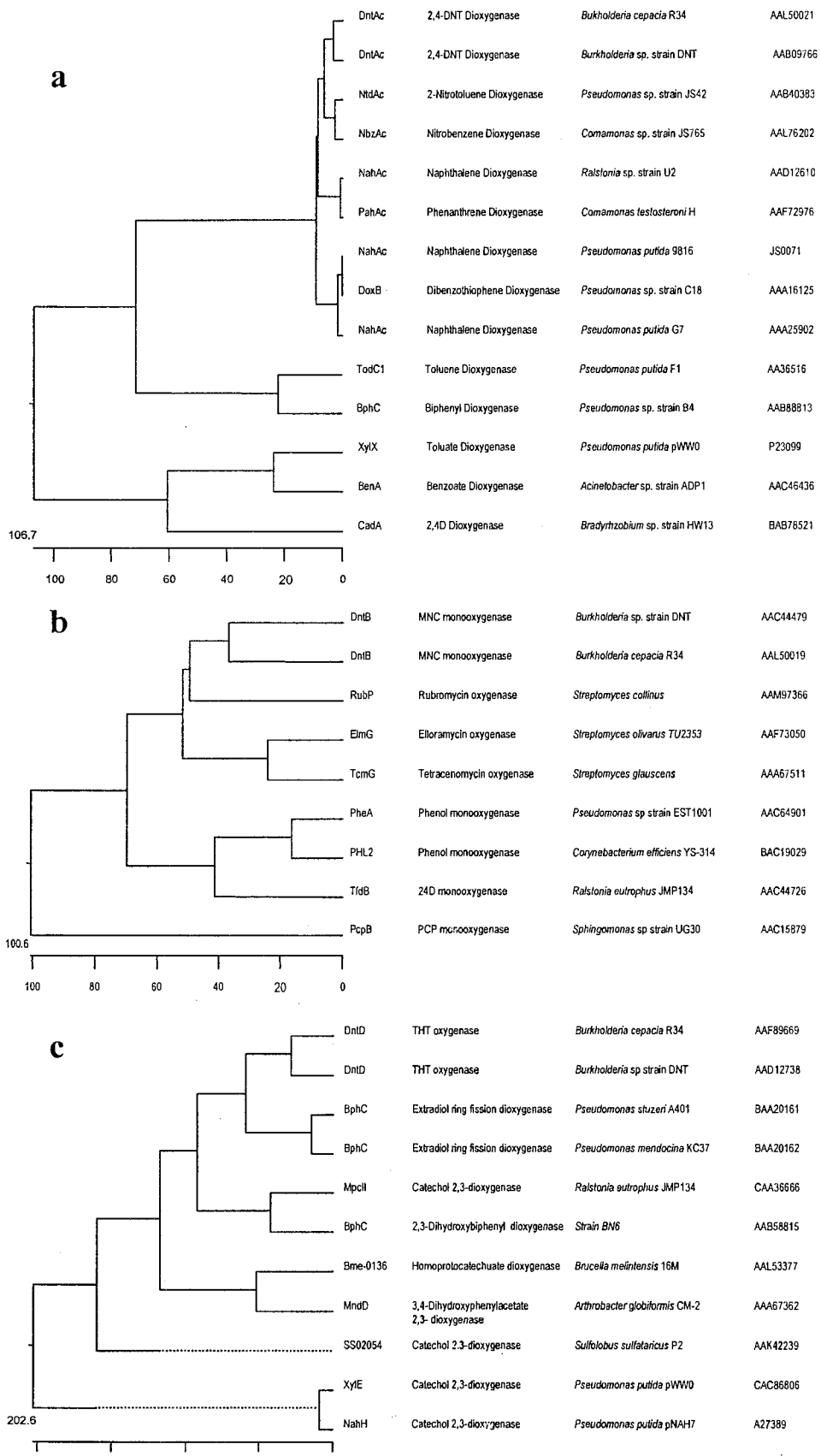

Fig. 2a–c Phylogenetic relationships of the oxygenases in 2,4-DNT degradation and homologues selected from results of a BlastP (Altschul et al. 1990) search of GenBank. Phylogenetic trees were generated using the MegAlign analysis tools in the Lasergene 99 software package (DNASTAR, Madison, Wis.). Multiple sequence alignment was done using the Clustal method within MegAlign. Gap and gap-length penalties were set at 10. The length of each branch pair represents the distance between the sequences. The scale below the tree indicates the number of substitution events between the sequence pairs. **a** α-Subunit of ring-hydroxylating dioxygenases. **b** Flavin-monooxygenases. **c** Ring-fission dioxygenases

hydroxylase homologues within the nitroarene dioxygenase operons (Parales et al. 1996; Suen et al. 1996; Fuenmayor et al. 1998; Johnson et al. 2002; Lessner et al. 2002) clearly indicates recent divergence from a gene cluster similar to that encoding the naphthalene dioxygenase from strain U2. The salicylate hydroxylase does not catalyze a step in any of the nitroarene degradation pathways, so genetic drift that eliminates the function will not be detrimental to the nitroarene-degrading organism.

The 4-methyl-5-nitrocatechol that results from dioxygenation of 2,4-DNT is subject to attack by methylnitrocatechol (MNC)-monooxygenase, a flavoprotein encoded by *dntB* (Fig. 1). The MNC-monooxygenase catalyzes oxidative removal of the nitro group and formation of 2-hydroxy-5-methyl benzoquinone (Haigler et al. 1994). The reaction is similar to the monooxygenase-catalyzed hydroxylation in 4-nitrophenol degradation, which yields benzoquinone and nitrite (Spain and Gibson 1991; Hanne et al. 1993), and to numerous other flavin-monooxygenases that oxidize hydroxy-nitroaromatic compounds as substrate analogues (Zylstra et al. 2000). All of the nitroarene monooxygenases studied to date are soluble enzymes, except the membrane-associated 4-nitrophenol monooxygenase from a *Moraxella* sp. strain (Spain and Gibson 1991) and all use NADH or NADPH as a cofactor in the reaction. Thus, the basic mechanism and biochemistry of the MNC-monooxygenases and other nitroarene monooxygenases appear to be similar, but more detailed molecular characterization of the enzymes is needed to determine how the present capabilities evolved.

The MNC-monooxygenases are only distantly related to monooxygenases that act on other aromatic compounds (Haigler et al. 1996; Johnson et al. 2002; Fig. 2b). They are more closely related to monooxygenases associated with antibiotic biosynthesis in *Streptomyces* spp than to monooxygenases from catabolic pathways (Fig. 2b). The flavin-monooxygenases from other catabolic pathways for nitroaromatic compounds are not closely related to MNC-monooxygenase. Preliminary reports on the *para*-nitrophenol (PNP)-monooxygenase from *Pseudomonas* sp. strain ENV2030 suggest that PNP- and MNC-monooxygenases are not homologous (Zylstra et al. 2000). Another potential homologue of the MNC-monooxygenases is the pentachlorophenol (PCP)-4-monooxygenase (PcpB) from *Sphingomonas* spp strains. Some PCP-degrading *Sphingomonas* strains appear also to mineralize PNP (Leung et al. 1997). PcpB has limited activity against PNP, but readily transforms 4-nitrocatechol to yield nitrite and a polar aromatic compound, presumed to be 1,2,4-benzenetriol (Leung et al. 1999). The reactions catalyzed by PcpB are analogous to the MNC-monooxygenase (Haigler et al. 1996; Fig. 1). Nonetheless, the amino acid sequences of the PCP- and MNC-monooxygenases are not closely related (26–30% identical), although each retains sequence motifs associated with NAD(P)H-dependent flavin-monooxygenases (Haigler et al. 1996; Johnson et al. 2002). No other 4-nitrocatechol monooxygenase sequences are available for comparison.

It will be surprising if the MNC-monooxygenases and 4-nitrocatechol-monooxygenases are closely related. The MNC-monooxygenases from strains DNT and R34 share only 53% identity (primarily in the N-terminal half of the polypeptide; Johnson et al. 2002) and are clearly distinct from other flavin monooxygenases that oxidize nitroarenes. The phylogenetic distance between the MNC-monooxygenases and other known flavin-monooxygenases complicates speculation on their origin. The context of the MNC-monooxygenase gene from strain R34 suggests recruitment from a catabolic pathway. A gene encoding a complete benzenetriol intradiol ring-fission dioxygenase is immediately downstream from the MNC-monooxygenase gene. Intradiol ring-fission dioxygenases are generally used in pathways for chloroaromatic compounds. The association of the MNC-monooxygenase and vestigial (with respect to 2,4-DNT degradation) ring-fission enzyme strongly suggests recruitment from an operon for chlorophenol degradation. Yet, the sequence comparisons indicate closer relatives among monooxygenases used in antibiotic biosynthesis (Fig. 2b).

The third module recruited for 2,4-DNT degradation encodes the lower pathway. Both the physiological (pathway) and genetic context of the trihydroxytoluene (THT)-oxygenase gene in strain R34 (Johnson et al. 2002) shed some light on the possible origin of the lower pathway for 2,4-DNT degradation. The THT-oxygenase of the 2,4-DNT pathway (Fig. 1), like the MNC-monooxygenase, has a narrow substrate preference and is distantly related to other catabolic enzymes (Fig. 2c; Haigler et al. 1999; Johnson et al. 2000). Moreover, the two THT-oxygenase polypeptides (DntD) from strains DNT and R34 share only 60% identity. The difference is significant, considering that ring-fission dioxygenases that have similar substrate preferences, e.g., catechol 2,3-dioxygenases are generally closely related (Eltis and Bolin 1996). The dissimilarity suggests an ancient common ancestor for the two characterized THT-oxygenases. It would be valuable to have molecular data for THT-oxygenases from additional 2,4-DNT-degrading strains and the THT-oxygenases used by *P. putida* ORC and *P. putida* O1OC in *meta*-cleavage pathways for degradation of resorcinol and orcinol (Chapman and Ribbons 1976a, 1976b). Comparisons between DntD and the dioxygenases with similar substrate preferences might provide more clues to the progenitor of the THT-oxygenases.

The steps that ultimately yield pyruvate and methylmalonyl-CoA are catalyzed by enzymes similar to those in pathways for degradation of amino acids (Johnson et al. 2002). In addition, a gene cluster encoding a putative ABC-transport complex for branched-chain amino acid transport is located upstream from the THT-oxygenase gene. However, the identity shared by the *dnt* genes and the putative homologues is not high enough to imply that the genes for the 2,4-DNT lower pathway were recently recruited from an extant pathway for amino acid catabolism. The *meta*- ring-fission dioxygenases like DntD are part of the vicinal oxygen chelate (VOC) superfamily,

which is defined by its conservation of a structural motif that allows coordination of a divalent metal ion (Armstrong 2000). The VOC superfamily includes extradiol dioxygenases, methylmalonyl-CoA epimerase, bleomycin- and fosfomycin-resistance proteins, and lactoylglutathione lyase (glyoxalase I). Accumulation of additional sequence information and identification of other biological pathways that include trihydroxytoluene will be the keys to defining the origin of the final steps in 2,4-DNT degradation.

Both of the 2,4-DNT pathways that have been genetically characterized are plasmid-borne (Suen and Spain 1993; Johnson et al. 2002), which provides a likely mechanism for horizontal distribution of the trait. Strains DNT and R34 were obtained from geographically isolated ecosystems and isolated within a decade of one another (Spanggord et al. 1991; Nishino et al. 2000a). It is possible that distribution of bacterial strains and genetic elements can occur, but the organization and sequence conservation of the genes encoding the 2,4-DNT pathways in strains DNT and R34 clearly indicate that their presence in the two strains did not result from recent horizontal gene transfer but rather from parallel evolution from common ancestors (Haigler et al. 1996, 1999; Suen et al. 1996; Johnson et al. 2000, 2002). If a simple conjugative transfer of the 2,4-DNT pathway genes from a common donor had provided the pathway to strains R34 and DNT, the organization of the genes would be similar and higher sequence identity would be expected. The genes for the 2,4-DNT pathway in strain R34 are contained within a 20-kb region (Johnson et al. 2002), but the genes that encode the pathway from strain DNT are distributed around the catabolic plasmid, pJS1 (Suen and Spain 1993). Moreover, the genes encoding MNC-monooxygenase and THT-oxygenase in each strain have quite different sequences considering that they catalyze identical reactions. Other 2,4-DNT degrading strains have been isolated from a variety of other sites contaminated with dinitrotoluene (Nishino et al. 2000a). Phylogenetic analysis of the genes encoding 2,4-DNT degradation in the other isolates might uncover evidence of horizontal transmission of the trait, or indicate independent evolution of the pathway in each geographically isolated site. Other questions still remain about how the individual steps in the pathways came together in strains DNT and R34. There is some evidence of transposition near the structural genes, but its contribution to the evolution of the pathways is unknown. Further characterization of the plasmids carrying the 2,4-DNT pathways will provide insight about the role of the plasmid in evolution of 2,4-DNT degradation and dissemination of the trait.

Nitrobenzene degradation: the partial reductive pathway

Most bacteria that mineralize nitrobenzene use a partial reductive pathway for aerobic degradation (Fig. 3). The first step is the reduction of the nitro group to hydrox-

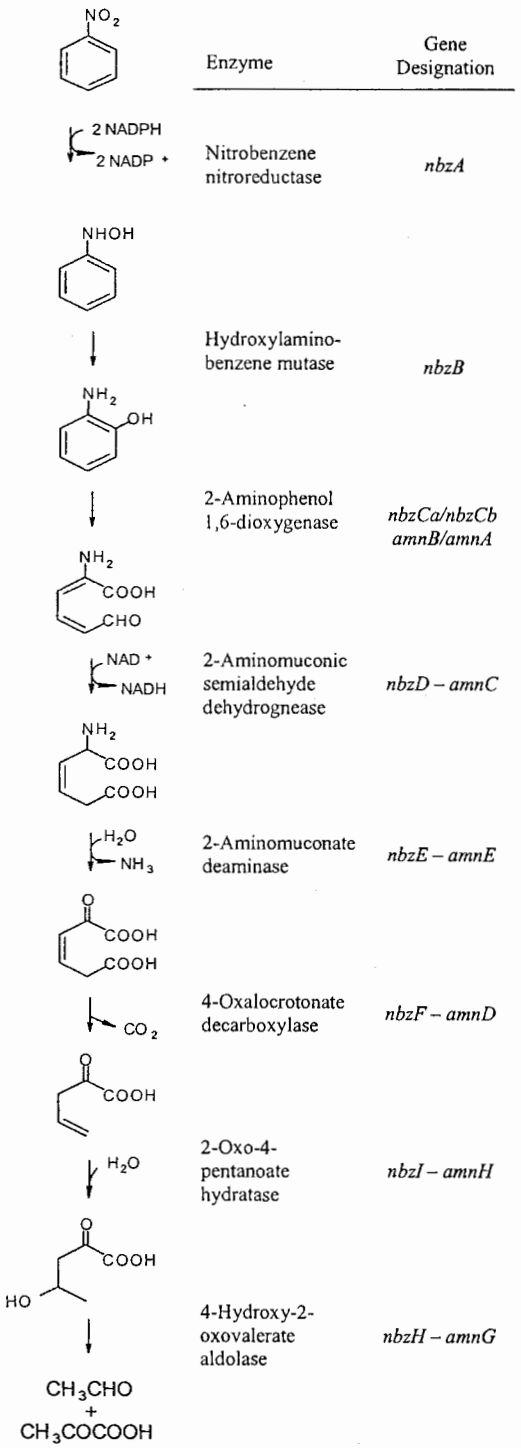

Fig. 3 Pathway for bacterial nitrobenzene degradation. Enzymes encoded by genes in *Pseudomonas putida* PS12, *P. pseudoalcaligenes* JS45, and *Pseudomonas* sp. AP3 are noted next to the reaction *arrows*. Only nitrobenzene nitroreductase, hydroxylaminobenzene mutase, aminophenol dioxygenase, and the 5′ end of the 2-aminomuconic semialdehyde dehydrogenase were characterized from strain JS45

ylaminobenzene, which is enzymatically rearranged to yield 2-aminophenal (AP). The 2-AP serves as the substrate for oxygenase-catalyzed *meta*- ring-fission. Following ring-fission, the pathway resembles the *meta*-ring-fission pathway for catechol degradation, except that enzymes of the downstream pathway also make accommodations for the amino substituent. The current understanding of the pathway results from studies of *P. pseudoalcaligenes* JS45 (Nishino and Spain 1993; Somerville et al. 1995; Lendenmann and Spain 1996; He and Spain 1997, 1998, 1999; He et al. 1998; Davis et al. 2000; Nadeau et al. 2003) and *P. putida* HS12 (Park and Kim 2000, 2001). Strain JS45 was isolated from soil taken from a nitrobenzene-manufacturing facility in the United States (Nishino and Spain 1993). Strain HS12 was isolated from nitroarene-contaminated soil in Korea, but the source was not reported (Park et al. 1999).

Substantial portions of the regions encoding the nitrobenzene pathways were cloned and sequenced from *P. pseudoalcaligenes* JS45 and *P. putida* HS12, as were those for the closely related pathway from the 2-AP-degrading *Pseudomonas* sp. strain AP-3, isolated by researchers in Japan (Takenaka et al. 2000). Comparison of the sequences and biochemistry provides a clear idea of the origin of the genes encoding ring-fission and downstream steps, but the progenitors for the enzymes that catalyze the initial steps in the pathway are not apparent.

The reactions catalyzed by the nitrobenzene nitroreductase are critical for the partial reduction of the molecule during nitrobenzene metabolism. The nitrobenzene nitroreductase from the nitrobenzene biodegradation pathway catalyzes the four-electron reduction of nitrobenzene stoichiometrically to hydroxylaminobenzene. If reduction of the hydroxylaminobenzene continued to the amine, subsequent rearrangement to AP would be impossible. Hydroxylaminobenzene is not a substrate for the reductase, so no aniline is produced (Nishino and Spain 1993; Somerville et al. 1995). The kinetic properties of the enzyme and regulation of its synthesis clearly indicate that nitrobenzene is the physiological substrate of the nitroreductase from strain JS45. The K_m of the nitrobenzene nitroreductase for nitrobenzene is much lower than those reported for the nitroreductases from enteric bacteria. Synthesis of nitrobenzene nitroreductase is inducible by nitrobenzene exposure (Nishino and Spain 1993), which suggests that the enzyme and its regulation evolved for nitrobenzene transformation. Based on the substrate preferences, it seems that the nitro group reductions catalyzed by the enzymes from enteric bacteria, e.g., *Escherichia coli* and *Enterobacter cloacae* (Bryant and DeLuca 1991; Koder and Miller 1998), are gratuitous reactions. A more likely physiological role of the nitroreductases from the enteric strains is quinone reduction (Cerniglia and Somerville 1995).

Comprehensive sequence information about nitrobenzene nitroreductases from bacteria that utilize nitrobenzene is limited to the *nbzA* gene from a catabolic plasmid carried by *P. putida* HS12 (accession AF319591; Park et al. 1999; Park and Kim 2000). NbzA has limited similarity to flavoprotein reductases available in GenBank (Fig. 4a), although the nitroreductase gene from strain JS45 was recently sequenced; and it shares 93% identity with NbzA from strain HS12 (J.-C. Chae and G. Zylstra, personal communication). Nitroarene nitroreductases from other catabolic pathways [the 4-nitrobenzoate nitroreductases from *P. putida* TW3 (Hughes and Williams 2001) and *R. pickettii* YH105 (accession AF187879)] share far less identity (Fig. 4a). Hypothetical proteins from several bacterial genomes share relatedness comparable to that of the 4-nitrobenzoate nitroreductase. It cannot be determined whether the gene products are nitroreductases for catabolic pathways or other flavin-dependent oxidoreductases. The conserved sequence of the two nitrobenzene nitroreductases from strains HS12 and JS45 and the favorable kinetic measurements suggest that the enzymes are highly specialized for nitrobenzene transformation and that the two enzymes share a recent common ancestor. The selective pressure that led to the present nitrobenzene nitroreductases is a mystery. Environmental exposure to nitrobenzene might not have been long enough to allow stochastic genetic drift and selection for evolution of a nitrobenzene nitroreductase. Conversely, a naturally occurring substrate that effects selection for the enzyme is not apparent either. For example, the nitroreductase from strain JS45 reduces the nitro- substituent of chloramphenicol, but it is not known whether this is a gratuitous reaction or a resistance strategy for the organism (L. Nadeau, personal communication). Examination of the nitroreductase genes and associated regions from other nitrobenzene-degrading bacteria and other bacterial genomes might provide additional insight and genetic context that indicates a role for the enzyme in other biochemical pathways and reveal a likely origin.

The second step in the nitrobenzene pathway, rearrangement of hydroxylaminobenzene to 2-AP (Fig. 3), is catalyzed by hydroxylaminobenzene mutase. Reactions involving rearrangements of phenylhydroxylamines are found in other nitroarene degradation pathways, including the 4-nitrotoluene pathway from *Mycobacterium* sp. strain HL4-NT-1 (Spiess et al. 1998), the 3-nitrophenol and 2-chloro-5-nitrophenol pathways of *R. eutropha* JMP134 (Schenzle et al. 1999a, 1999b), the 4-chloronitrobenzene pathway from strain LW1 (Katsivela et al. 1999), and the 2-nitrobenzoate pathway from *P. fluorescens* KU-7 (Hasegawa et al. 2000). The examples discovered to date indicate that the rearrangement catalyzed by mutases is widely used for degradation of nitroaromatic compounds.

Hydroxylaminobenzene mutase genes from the nitrobenzene-degrading strains *P. putida* HS12 and *P. pseudoalcaligenes* JS45 are clearly homologous (Fig. 4b). Like the nitrobenzene nitroreductase, the mutase gene (*nbzB*) is also plasmid-borne in strain HS12, but on a separate plasmid from the reductase gene (Park and Kim 2000). Curiously, the chromosome of strain JS45 carries genes encoding two mutase isozymes (*habA*, *habB*). The two mutase genes are separated by 2.5 kb, share only 44% amino acid identity, and have significantly different G+C profiles (53%, 71%; Davis et al. 2000). The differences

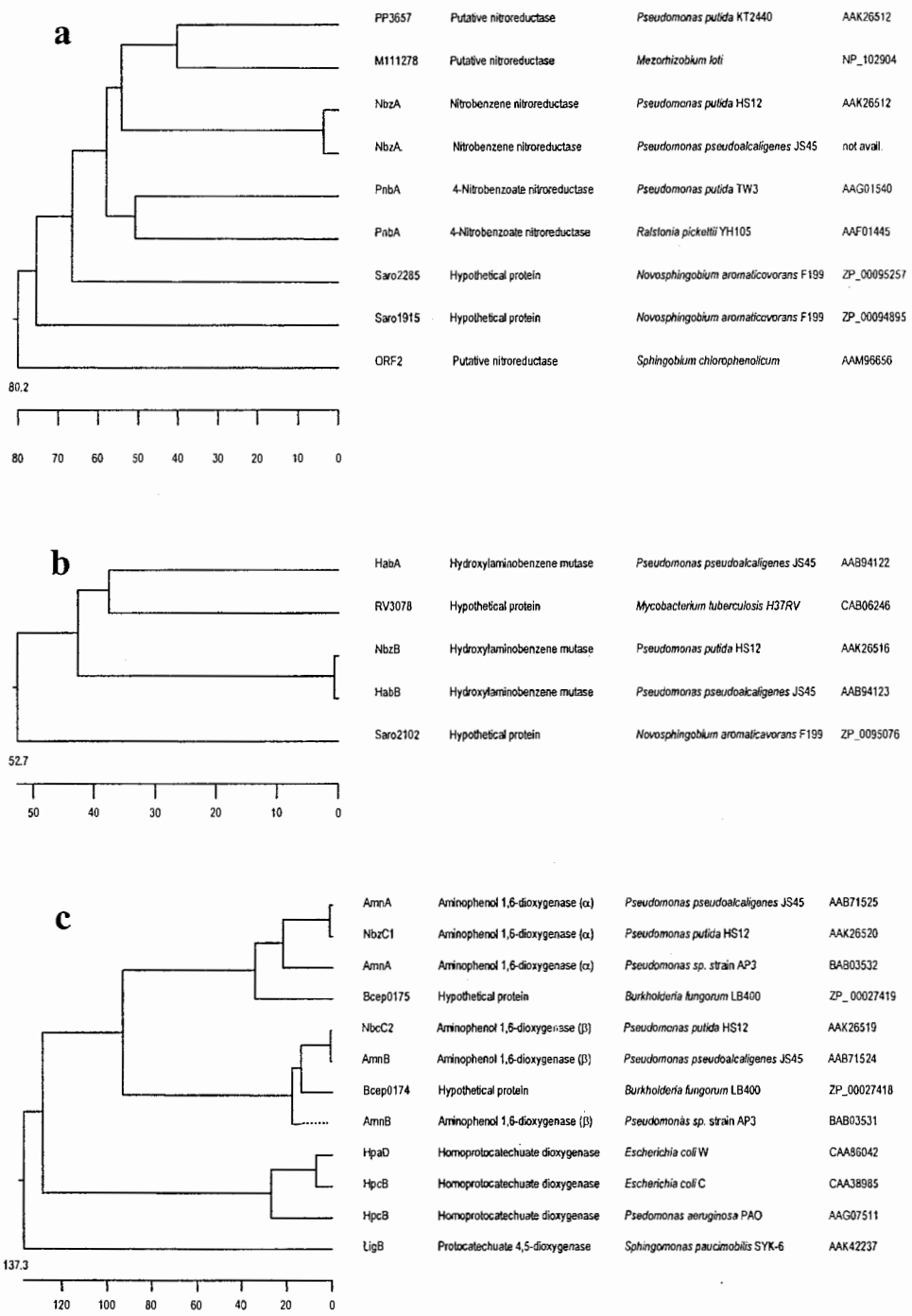

Fig. 4a–c Phylogenetic relationships of the enzymes for initial steps in nitrobenzene degradation and homologues selected from results of a BlastP (Altschul et al. 1990) search of GenBank. Phylogenetic analysis done as noted in Fig. 2. **a** Nitrobenzene nitroreductase. **b** Hydroxylaminobenzene mutase. **c** Aminophenol 1,6-dioxygenase (α-, β-subunits)

seem to rule out recent gene duplication. Both genes can encode a functional enzyme. Recombinant *Escherichia coli* strains carrying either gene expressed hydroxylaminobenzene mutase activity, but RNA analysis revealed that only *habA* is expressed in strain JS45 (Davis et al. 2000). The mutase from strain HS12, NbzB (GenBank accession AAK26516), shares 99% identity with the HabB isozyme from strain JS45, suggesting a very recent common ancestor. It is odd that the *habA* gene encodes the physiologically relevant isozyme for strain JS45, yet HabB is virtually identical to the mutase that strain HS12 uses in its nitrobenzene pathway. A survey of other nitrobenzene-degrading bacteria might reveal whether one of the mutase isozymes predominates among the nitrobenzene-degrading isolates.

A search of GenBank reveals five putative mutase genes (Fig. 4b): the two isozymes from strain JS45 (*habA*, *habB*), the mutase from strain HS12 (*nbzB*), and two

hypothetical proteins identified from genome-sequencing efforts. The first is from *M. tuberculosis* (GenBank accession CAB06246) and the second from *Novosphingobium aromaticivorans* F199 (GenBank accession ZP_0095076). The mycobacterium gene product has hydroxylaminobenzene mutase activity when expressed in recombinant *E. coli* strains (Davis et al. 2000). The *N. aromaticivorans* strain (formerly *S. aromaticivorans* F199) was isolated from a deep subsurface-sediment sample (Fredrickson et al. 1991) and is noted for its range of aromatic growth substrates and metabolic plasticity, although the ability to use nitrobenzene has not been reported. The context of the putative mutase gene from *N. aromaticivorans* is intriguing. A gene sharing significant identity with the nitrobenzene reductase from strain HS12 and the putative mutase gene appear to overlap in the *N. aromaticivorans* genome (accessions ZP_0094895, ZP_095076). The arrangement would yield a potential nitrobenzene upper-pathway cassette, i.e., the genes allowing for transformation of the nitroarene to the ring-fission substrate. Unlike the separation of the reductase and mutase genes in strains HS12 and JS45, the organization in strain F199 provides potential for efficient transfer of the genes to another host and regulation of expression.

AP-1,6-dioxygenase (AP-dioxygenase) catalyzes ring-fission in the nitrobenzene catabolic pathway (Fig. 3; Nishino and Spain 1993). The reaction differs from the ring-fission step in most pathways for degradation of aromatic compounds, which involve dihydroxy and trihydroxy compounds as ring-fission substrates. The biochemistry and molecular genetics were reported for AP-dioxygenases from two nitrobenzene-degrading strains [strain JS45 (Lendenmann and Spain 1996; Davis et al. 1999), strain HS12 (Park and Kim 2001)] and a 2-AP-degrading strain [*Pseudomonas* sp. strain AP-3 (Takenaka et al. 1998)]. The AP-dioxygenases are heterotetramers with an $\alpha_2\beta_2$ native configuration (Lendenmann and Spain 1996; Takenaka et al. 1997). The AP-dioxygenases have relatively narrow substrate ranges, which suggests that the enzymes are highly specialized for transformation of 2-AP (Lendenmann and Spain 1996; Takenaka et al. 1997).

The AP-dioxygenases are closely related to one another, but quite dissimilar to other ring-fission enzymes (Fig. 4c). The subunit sequences from the nitrobenzene-degrading strains are well conserved with 99% and 98% identity shared for the α- and β-subunits, respectively (Davis et al. 1999). The AP-dioxygenase genes from strain AP-3 are clearly homologous (84%, 67% identity) to those from the nitrobenzene-degrading strains but the point of divergence was much earlier (Fig. 4c). Another putative AP-dioxygenase is found in the genome of *B. fungorum* LB400 (formerly *Pseudomonas* sp. strain LB400; Fig. 4c), a strain noted for its ability to degrade polychlorinated biphenyls (Gibson et al. 1993). The discovery suggests that the AP pathway might be widely distributed. The next-closest relative to AP-dioxygenase is homoprotocatechuate dioxygenase (EC 1.13.11.15), which has a homotetrameric active form. The catalytic β-subunit of AP-dioxygenase shares limited identity with the homoprotocatechuate dioxygenase, from the widely distributed bacterial tyrosine degradation pathway (Fig. 4c).

A progenitor for the AP-dioxygenases among the dioxygenases for other aromatic compounds is not apparent. Comparisons of the AP-dioxygenase β-subunit sequences with the conserved domains database of GenBank (Marchler-Bauer et al. 2003) reveal that the β-subunit for the AP-dioxygenase is similar to LigB, the catalytic subunit of the protocatechuate-4,5-dioxygenase from lignin biodegradation pathways of numerous soil bacteria (Dagley 1986). Like the AP-dioxygenases, protocatechuate-dioxygenase pairs non-identical polypeptides (LigB, LigA) to yield the functional enzyme, but the basic structural analogies between AP-dioxygenase and protocatechuate-dioxygenase are deceptive. The α-subunits from AP-dioxygenase (NbzC1, AmnA) and protocatechuate-dioxygenase (LigA) are not related (Takenaka et al. 1997). The differences indicate that the protocatechuate- and AP-dioxygenases came about by different evolutionary routes. One plausible hypothesis is that the individual subunits of the AP-dioxygenase arose from ancient gene duplication (Takenaka et al. 1997); and this suggestion is supported by the fact that the α- and β-subunit sequences of AP-dioxygenase are more closely related to one another than to any other polypeptides in the database and are equally distant from their closest relative, the homoprotocatechuate-dioxygenase (Fig. 4c). In the native AP-dioxygenase, the β-subunit appears to contain the catalytic residues of the progenitor ring-fission dioxygenase (Takenaka et al. 1997; Davis et al. 1999; Park and Kim 2001) and the α-subunit is proposed to act as a stabilizer for the tetrameric enzyme structure (Takenaka et al. 1997).

The lower pathways for nitrobenzene degradation from strains JS45 and HS12 are remarkably similar, despite different geographic origins of the strains, and appear identical to the AP pathway in strain AP-3. The steps following ring-fission of 2-AP pathway are similar to the commonly used catechol *meta*- ring-fission pathway (Dagley 1986), with some modification. The hydrolytic branch of the *meta*- pathway is absent in the AP pathway and a deaminase is present in the AP pathway to allow transformation of 2-aminomuconate to 4-oxalocrotonate in the place of 4-oxalocrotonate tautomerase in the catechol pathway (He and Spain 1997, 1998; Takenaka et al. 1998; Park and Kim 2001). Biochemical comparisons show that the reactions of the deaminase and tautomerase take place at analogous points in the degradative pathway and the substrates have similar structures, which suggests that the enzymes are homologous (He and Spain 1998). Molecular characterization of the polypeptides does not corroborate the reaction analogy. The amino acid sequences for 2-aminomuconate deaminase and 4-oxalocrotonate tautomerase share no significant identity.

The genetic organization of the AP pathway appears to be well conserved and is identical with the two AP operons that have been completely sequenced (strains AP-3, HS12; Takenaka et al. 2000; Park and Kim 2001) and with the 5′ end of the operon characterized from strain JS45 (He et al. 1998; Davis et al. 1999). The conservation suggests there has been extended selective pressure to generate a pathway for 2-AP metabolism. 2-AP is a major industrial chemical that is manufactured and used as a synthetic intermediate, its use has undoubtedly led to widespread environmental contamination. One natural source of 2-AP is the breakdown of cyclic hydroxamic acids, antibiotics that grasses synthesize to protect against fungal infection. The principal hydroxamic acid made by rye grasses, 2,4-dihydroxy-1,4-benzoxazin-3-one, is transformed by fungal pathogens to yield 2-AP as a decomposition product (Morrissey and Osbourn 1999). Accordingly, bacterial exposure to 2-AP is not solely a result of contemporary nitrobenzene degradation and industrial 2-AP production/disposal. A natural source of the compound results from decomposition of plant secondary metabolites, a ubiquitous and much older process than modern synthetic chemistry. Perhaps the opportunity to utilize the naturally occurring 2-AP provided the scale and time-period that allowed evolution and distribution of an AP pathway. Studies of the microbial ecology and relevant molecular genetics of additional AP-degrading bacteria might determine the age and origin of the 2-AP pathway. The 2-AP degradation operon also provides an opportunity for vertical substrate expansion analogous to chlorobenzene degradation. The lower pathway joined with the nitroreductase and mutase would yield a degradative pathway for the nitrobenzene.

Conclusion

The current understanding of the evolution of pathways for bacterial nitroarene catabolism grew from detailed study of genes encoding selected enzymes in the pathways or regions encoding entire pathways. Characterization of additional isolates that degrade specific synthetic compounds can enhance the understanding of a few pathways but might not expand our comprehension of evolution in general or predict the fate of synthetic compounds in the environment. Questions remain about how the forces of selection were brought to bear on the systems that produced the pathways. Genomic studies might reveal precursors of relevant enzymes or show that the catabolic potential is more widely distributed than previously believed; but fundamental understanding of the relevant biochemistry and physiology is still critical to identify functions of the gene products.

The mechanisms involved in the evolution of catabolic pathways seem well established. Gene duplication and mutation allow for alteration or broadening of the substrate preference of the enzymes. Genes and gene clusters are recruited and assembled via various horizontal transfer mechanisms. Variant strains able to use the new substrate can proliferate and selective pressure allows strains carrying the best adapted enzymes (with respect to K_m, K_{cat}) to thrive with the new substrate. Further advantage is granted when organisms develop mechanisms that regulate the synthesis and function of the enzymes (Cases and de Lorenzo 2001). What is not known is when and where the appropriate conditions allow selection for a catabolic pathway. Microbiologists are regularly isolating bacteria that metabolize synthetic compounds that were previously deemed recalcitrant to biodegradation. Are these isolates the product of accelerated evolution, proliferation of existing bacteria in the ecosystem, or more experienced microbiologists? As in archeology, it is difficult to design definitive experiments to test the alternate hypotheses.

Acknowledgements We thank Gerben Zylstra and Lloyd Nadeau for sharing unpublished data and Shirley Nishino for critical review of the manuscript. The work was supported in part by the Air Force Office of Scientific Research and the Strategic Environmental Research and Development Program.

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